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It is widely presumed that environmental exposures play a role in the development of breast cancer, but only ionizing radiation has been identified as a risk factor for the disease. Individual susceptibility to the types of genotoxic damage and mutagenesis caused by ionizing radiation should therefore be a modulator of breast cancer risk. A number of inborn human disease syndromes characterized by ioning radiation sensitivity have been identified, including ataxia telangiectasia, which has also been linked with breast cancer risk through molecular and epidemiological studies. The V(D)J recombinase assay exploits the known association of specific chromosome aberrations with the AT phenotype; chromosome aberrations caused by the inappropriate "illegitimate" inter-recombination of elements of different genes that normally undergo V(D)J recombination during maturation of the immunological system, the immunoglobulin and T-cell receptor genes. This PCR-based assay has the advantages of being simple, rapid and relatively inexpensive, allowing for feasible application in population screening to detect individuals at increased risk of breast cancer prior to the onset of overt disease. This assay will now be applied within the larger context of a study measuring overall genotoxic exposure and response in newly diagnosed breast cancer patients using blood cell-based somatic mutation assays.

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## Introduction

About 10% of incident breast cancer can be attributed to hereditary factors, and a further 15% can be accounted for by a complex mix of life history factors widely interpreted as representing endogenous hormone production. This leaves the great majority of breast cancer unaccounted for. It is assumed that environmental exposures are involved in breast carcinogenesis, and strong evidence for the effect of radiation has been presented. Total genotoxic exposure, especially the contributions of the countless chemicals in the environment, are impossible to calculate based on the agents themselves. Such exposure monitoring also does not integrate the individual response to genotoxic exposure that modulates its effect on processes such as carcinogenesis. Ataxia telangiectasia (AT) is a human hereditary disease characterized by increased susceptibility to the genotoxic and cytotoxic effects of ionizing radiation. AT patients often exhibit an increased frequency of chromosome aberrations in their lymphocytes, particularly chromosome aberrations caused by illegitimate VDJ recombination, i.e., translocations caused by the splicing together of genes that normally undergo VDJ recombination during the development of the immune system. We have proposed that quantitation of such aberrations in the blood of newly diagnosed breast cancer patients could determine whether ionizing radiation exposure and sensitivity plays a role in the development of "sporadic" breast cancer. The current project is designed to provide pilot data on this hypothesis with retrospective data on 50 breast cancer patients, along with suitable controls for comparison.

## Body

Risk factors for the development of breast cancer remain largely unknown, however, several clear elements have emerged: family history of breast cancer, metabolic factors related to hormone production, and exposure to X irradiation (1,2). It has been suggested that breast cancer incidence is also influenced by the accumulation of man-made chemicals in the environment. Two types of environmental chemicals have been implicated; those that mimic hormonal effects, known as "xenoestrogens", and those that mimic the DNA-damaging effects of X irradiation, or "genotoxicants". We hypothesize that breast cancer incidence should be a product of both the total cumulative exposure to genotoxic agents, including but not limited to X-rays, as modified by differences in individual response to this exposure as mediated by factors such as metabolic detoxification (or activation) and DNA repair capacity.

Although there is bound to be some element of tissue specificity for both genotoxic exposure and susceptibility to DNA damage, it is impractical to monitor somatic mutation in breast tissue itself. Blood, however, and its progenitor tissue bone marrow, are present throughout the body, and most xenobiotic exposures to the breast are likely to be transported to the breast tissue through the blood (3). Moreover, blood also carries B and T lymphocytes, components of the bodily immune system that uniquely splice the DNA of their T-cell receptor or immunoglobulin genes into unique patterns. Thus, these cells are able to manifest genetic damage occurring via illegitimate V(D)J recombination (4), a mutagenic process that is characteristic of loss of double strand break DNA repair, such as is characteristic of the cancer-prone syndrome AT. The

*BRCA1* and *BRCA2* breast cancer predisposition genes have also been implicated in this type of repair, so may also have an intrinsic susceptibility to these types of mutations (5).

Our major accomplishment of 2001 was the creation of a new IRB protocol acceptable to both our local committee and to the DOD. This was accomplished by nesting the current pilot project within a larger study (DAMD17-00-1-0409) with a pair of more generalizable biomarkers: somatic mutation at the *GPA* locus in erythrocytes and the *HPRT* locus in erythrocytes. The combined study will provide information on the possible influences of ionizing radiation exposure and susceptibility on the frequency and spectrum of somatic mutation. IRB approval for the larger study was granted May 23, 2002, and the revised protocol including analysis of VDJ recombination was approved by the IRB of Magee-Womens Hospital on October 28, 2002. Accumulation of patient samples through this larger protocol was significantly delayed by the addititon of new HIPAA regulations, but is ongoing and should eventually provide more than the original 50 patients requested for the pilot study. We have taken the opportunity to develop a pair of complementary molecular assays that will better characterize the specificity of the illegitimate VDJ recombination occurring in our patients.

## Key Research Accomplishments

- We have completed an IRB protocol acceptable to both our local (Magee-Womens Hospital) committee and the DOD.
- We have established that our route to patient sampling is and remains viable, and have begun to accumulate patient and control DNA samples for analysis.
- We have established that our assay technologies are still workable and can be passed on to new workers in the lab.
- We have identified a new source of samples from AT patients (positive controls for the VDJ recombination assay) and entered into a collaboration with Dr. Blanche Alter of the NCI.
- We have extended the analysis from the original translocation site to two more recurrent translocations in AT that suggest different degrees of specificity with respect to the translocation signal.

## Reportable Outcomes

As the work is pending, all reportable outcomes are still in the future. The original biomarker proposed for this study was based on PCR amplification of a novel product across a characteristic recurrent translocation found in AT patients (6,7), but also inducible by

environmental exposures (8). We generated the primers necessary for this analysis, then found that the cell line reported to carry this translocation (6,9) was unstable and had been lost. We were unable to demonstrate the expected translocation amplification product in a number of AT-derived cell lines from the Coriell Cell Repository, but have arranged a collaboration with Dr. Blanche Alter of the NCI to obtain fresh blood samples from AT patients.

We have also developed two other PCR-based assays for illegitimate VDJ recombination. Whereas the inv(7) chromosomal marker involves the splicing together of two immune function genes, thus utilizing bona fide splicing signals albeit incorrectly, these new markers involve splicing at one, or both, cryptic signals elsewhere in the genome.

The first new marker is based on a t(14;18) chromosomal translocation found in approximately 85% of follicular lymphomas by both cytogenetic and molecular analyses (10,11). This rearrangement deregulates expression of the *BCL-2* oncogene by juxtaposition into the *Ig* heavy chain locus by illegitimate V(D)J recombination (12). A sensitive PCR-based assay has been developed for the detection of this translocation in peripheral blood lymphocytes, and has demonstrated that the t(14;18) can be detected at low levels in almost all healthy individuals (13,14).

The second new marker is based on a characteristic deletion of exons 2 and 3 in the X-linked *HPRT* gene that is also mediated by illegitimate V(D)J recombination. This deletion was first discovered in cord blood lymphocytes (15) and was subsequently found in adults (16). A quantitative PCR assay was developed to measure the frequency of this deletion in peripheral blood cells of humans, where it was found to range from $< 1.3 \times 10^{-7}$ to $4.1 \times 10^{-7}$ (14,17). This illustrates another benefit of adding these two markers to the study; these studies have developed a means of deriving mutant frequencies from these biomarkers instead of just the semi-quantitative method used with the original marker (8). This second marker is also part of the molecular analysis of the mutations contributing to the *HPRT* mutation frequency assay, one of the two main assays in the larger study.

This larger study consists of the application of the *GPA* and *HPRT* somatic mutation assays on 200 newly diagnosed breast cancer patients and controls. Preliminary data (18; included in the Appendix) suggests that breast cancer patients have significantly higher burdens of somatic mutation in their bone marrow, but it is unclear whether this is due to genotoxic exposures or genetic susceptibility (or both). The addition of these PCR-based biomarkers for illegitimate VDJ recombination to this study will begin to address this question by providing an independent measure of double-strand break damage and repair. The application of the VDJ biomarkers will also be enhanced by the context of the greater study, in that traditional risk factors, including family cancer incidence, lifestyle factors associated with estrogen exposure and some environmental exposures are collected. In addition, with the expertise and resources developed under the present grant, we will be able to go beyond the initial pilot study of 50 patients if the results warrant it.

Thus, our eventual reportable outcomes should provide confirmation that exposure biomarkers are associated with breast cancer, and how they are modulated by genetic susceptibility, specifically to DNA double-strand breaks. We should be able to demonstrate how

our exposure biomarkers interact with the known predictive factors of family history and life history of estrogen exposure. Finally, we will be able to determine whether a specific type of exposure, ionizing radiation and chemical mimics of its chromosome breaking effects, is implicated in breast carcinogenesis in an unselected general patient population, which could then allow for the development of preventive, anti-cancer agents, and, perhaps, allow for the further individualization of cancer chemotherapy.

## Conclusions

Our somatic mutation data (18, and unpublished) suggest that environmental exposure is a powerful factor in the development of breast cancer, but that it has too many individual contributors to monitor a single genotoxic agent. A particular type of damage implicated from risk analysis is exposure to ionizing radiation. The current study will allow for the quantitative monitoring of ionizing radiation-like DNA damage and susceptibility, within the context of a larger study with less specific mutational biomarkers. With IRB and HIPAA concerns behind us, we are ready to get to the job of confirming and extyending our initial studies with regard to delineating the differences between those who develop breast cancer at a certain age, and those who do not.

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## Appendices

Grant SG (2001) Molecular epidemiology of human cancer: biomarkers of genotoxic exposure and susceptibility. J Environ Pathol Toxicol Oncol 20: 245-261.

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# Molecular Epidemiology of Human Cancer: Biomarkers of Genotoxic Exposure and Susceptibility

*Stephen G. Grant*

The new field of molecular epidemiology investigates the link between toxic exposure and an associated health effect by defining presumptive intermediate stages in the development of the disease based on known mechanisms. In the development of malignancy, these steps may involve exposure to known mutagens and carcinogens, internalization and potential metabolism of a chemical agent, characterization of the interaction of the agent at its site of action (usually DNA), characterization of induced preneoplastic changes, and, in certain instances, early detection of the cancer itself. These processes can be monitored through biomarkers specific to each of the steps in the progression toward disease using any of the host of applicable techniques now available. An overview of such techniques is presented, with emphasis on techniques offering insight into the malignant process. Evidence is presented suggesting that although there are many potential contributing mechanisms to carcinogenesis, mutagenesis remains the dominant driving force behind the process. Several methods of monitoring mutation have shown promise as predictors of cancer incidence. These methods might also be used as monitors of agents designed to intervene in the process to prevent the development of overt disease.

KEYWORDS: genetic toxicology, carcinogenesis, mutagenesis, GPA, HPRT, DNA repair, mutagen sensitivity

## Introduction

It has become fashionable to place the word *molecular* before the name of a classical field of scientific research and consider it reinvented. This often happens in the absence of what the word means in this context and how this refined and redefined field truly differs from its progenitor. In the case of molecular toxicology, there has been a real shift from the traditional activity of testing chemical toxicity in model systems to studies in the true organisms of interest, humans. These studies have their own advantages and disadvantages; they are, by definition, epidemiological, and epidemiology is very different from experimental science, requiring larger, more expensive and more interdisciplinary studies. Often the investigator has no control over the agent of exposure or the dose or doses administered; in most cases, one must rely on "found" experiments, such as accidental exposures, which are often uncomfortably similar to ambulance chasing. For the accumulation of significant data, more than an anecdotal case report is required, so there must be a relatively large exposed population with a consid-

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erable increase in the incidence of the disease. Indeed, this field often relies upon the pharmaceutical industry to provide a large population of exposed individuals exhibiting unanticipated toxic effects. This relative inability to study an agent of the investigator's choice is offset by the fact that real human beings are the source of data and there is no question of the applicability of the model system. These studies, therefore, involve what has become known in biomedical science as *translational* research, that is, science that has direct application to real-life situations.

In public health, the promise of molecular toxicology and molecular epidemiology is the identification of an impending disease before clinical manifestation, which potentially allows for biological, chemical, or behavioral intervention and, perhaps, prevention. This is a particularly appropriate approach to cancer because many avenues of research have shown carcinogenesis to be a multistep process with a duration or gestation time of decades. Cancer can result from the delayed effects of a single short-term exposure, such as a radiation accident, or from the effects of an otherwise asymptomatic chronic exposure. In this delayed or accumulative aspect of its etiology, it is very possible that cancer can act as a paradigm for other late-onset diseases because somatic effects are more important factors in the development of the disease than genetic predisposition. With the impending completion of the human genome project, however, attention has increasingly moved toward these genetic factors, even in diseases of aging. Besides the many technological tools being developed in this area, such as gene expression and polymorphism chips, the main reason for concentrating on genetics is that it can be fully ascertained at any age. For example, a blood sample from an 80-year-old contains all the genetic information that would have been available had the subject been sampled at birth. In contrast, toxicological exposures wax and wane, overlay one another, and are ongoing at any point of sampling; thus, there is no easily obtainable record of an exposure history similar to that of the underlying genetic background.

## Molecular Epidemiology

In the classical toxicological epidemiology model, a defined health effect, often a well-characterized clinical disease, is perceived as occurring from the exposure of an organism to a deleterious biological, chemical, or physical agent. This strict cause-and-effect relationship is mediated through a number of unknown modifiers of exposure and response related to the anatomy, biochemistry, and physiology of the organism. The molecular epidemiological model, as shown in Figure 1, attempts to expand on the concept of such biological modification by breaking the process into sequential stages that must be traversed to manifest disease. These intermediate stages are based on mechanistic studies and hypotheses that attempt to identify the target tissue or cell type (which may not be the same as the cell type affected by the disease, or even at the same site as the eventual disease manifestation), the response or responses necessary to convert exposure into biological effect, and, if possible, the preclinical evidence of impending disease. As in any hypothetical system, experimentally verifiable predictions indicative of each stage are important. These indicators of biological modification are known as *biomarkers* and, because they precede clinical disease, they are thought of as *intermediate* biomarkers that can be used to monitor the progress of the disease.

The development of the field of molecular epidemiology has been, and continues to be, hindered by a lack of complete understanding and cooperation between the practitioners of the two progenitor disciplines, laboratory toxicologists and epidemiologists. For the toxicologist, the traditional laboratory truism, "If you need statistics to prove your point, you didn't design the experiment properly," is difficult to reconcile with epidemiological studies. On the other hand, epidemiologists, especially clinical epidemiologists, often seem to forget that statistical associations are not and cannot be proofs of causality. The proper course is for epidemiological studies to generate mechanistic hypotheses that are then evaluated experimentally. Too often, there is a complete disconnection between the two disciplines. Epidemiologists hire technicians to perform tests they have seen published in the literature, often without thoroughly understanding the relevance or implications of the results. Toxicologists, however, attempt to apply their knowledge of experimental design to epidemiological studies without appreciating the statistical methodologies necessary to adjust for unanticipated, confounding effects. In many ways,

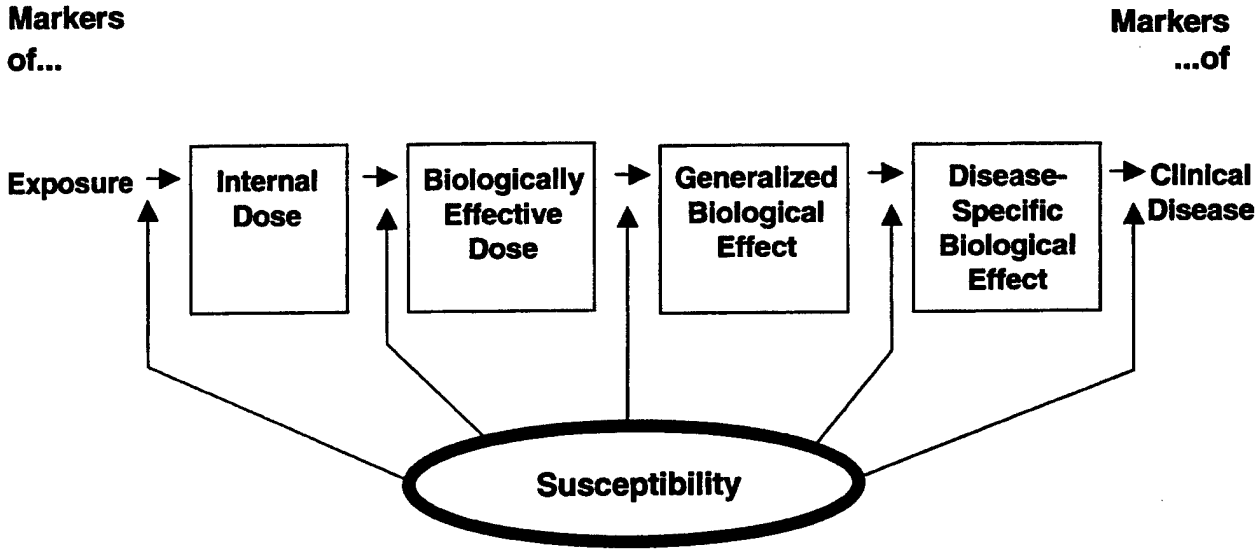


FIGURE 1. Epidemiology of induced human disease in the mechanistic context of molecular toxicology. Insights into the absorption, distribution, metabolism, and elimination of environmental agents are combined with insights into the mechanism of the disease process to provide potential intermediate steps in the progression that can be tested for validity and applied as surrogates for the eventual health effect.[1–4]

the situation is reminiscent of the difference between academic and industrial or regulatory toxicologists: Academic toxicologists apply a continually revised or "improved" protocol to a series of individual, often unrelated projects, whereas industrial and regulatory toxicologists apply a standardized, but almost always obsolete or suboptimal protocol, to a very systematic study of an area of proven concern. Thus, in collaboration, the laboratory toxicologist can address the mechanistic relevance of a biomarker to the disease of interest, troubleshoot, and adapt the protocol to the types of samples that can be obtained, and offer the possibility of experimental follow-up on mechanistic hypotheses that might result from an epidemiological study. The epidemiologist, in turn, directs the study to a question of immediate concern to medicine or public health and allows for testing of both a mechanistic hypothesis and the biomarker designed to detect and monitor it in human studies.

## Biomarkers of Carcinogenesis

To propose and test biomarkers of a specific disease, some insight into its etiology must be available. There have been many models of the carcinogenic process proposed: epigenetic, viral, toxicological, endocrine, immune surveillance, histopathological, and so forth, but the somatic mutational model[5] has become predominant for several reasons. First, there was the discovery of dominant activated oncogenes and recessive tumor suppressor genes and their identification in all types of cancer.[6,7] Second was the linking of these mutations with histological progression, as best exemplified by the Vogelstein et al.[8] model of colorectal cancer. Third, there is the unique ability (and willingness) of the supporters of this model to integrate aspects of other models into itself. For example, the somatic mutational model has to be compatible with the viral model because activated oncogenes were first identified in oncogenic viruses, and only subsequently were shown to have homologues in the host genome. The model is also flexible enough to allow that epigenetic changes in gene expression, such as endocrine stimulation, hyper- or hypomethylation of genes, can have the same effect as mutation in fulfilling the requirements of a step in the carcinogenic pathway. Toxicologists are satisfied with the mutational model because it describes a multistep process involving classical mutations that can be caused by

radiation or electrophilic chemicals. Thus, most intermediate biomarkers of cancer presume that mutation is the only or principal mechanism of carcinogenesis and are designed to detect mutagenic exposures, premutagenic, and mutagenic lesions, as well as the biological effects of somatic mutations. Toxicologists must be reminded that not all cancer researchers are prepared to directly equate carcinogenesis with mutagenesis, despite the fact that this principle underlies almost all carcinogenicity testing and costs industry billions of dollars.

## Carcinogenic Exposure

Practically, there are two approaches for studying carcinogenic exposures: identification of actual exposures and identification of potential exposures. Obviously, the former is often retrospective, whereas the latter is prospective. Applied primarily to anthropogenic chemicals, a large number of carcinogenicity, mutagenicity, and other types of assays have been developed to determine or predict whether a chemical is a potential human carcinogen. The gold standard is the chronic animal cancer test; rodent carcinogenicity tests are the most widely applied.[9] These lifetime studies are time-consuming and expensive, often have questionable application to humans, and have been increasingly criticized by animal-rights activists. Attempts to establish single-cell short-term assays have usually been based on a mutational approach to the carcinogenic process, and measured genotoxicity.[10] All of these tests suffer from fundamental oversimplifications in their basic assumptions. For example, they must assume that biological effects of exposures to multiple genotoxicants (including all in vivo exposures) can be estimated from additively combining the efficacy of individual constituents, which suggests that interactions such as synergism and antagonism either do not occur, or, overall, balance one another out. They also must assume that all genotoxicants have simple dose–response kinetics, which ignores the possibilities of hormesis or other higher-order interactions. Presently, a huge number of manufactured chemicals in use lack significant toxicological data; however, there are at least two promising approaches toward improving testing efficiency in the near future. First is the adoption of high-throughput and high-content screening technologies, using advances in fields such as robotics, flow cytometry, computer-directed microscopy, mass spectroscopy, and so forth, to better apply our knowledge of carcinogenesis. Such technologies have already been successfully applied in some aspects of toxicology,[11–14] but not to the degree they have been embraced by pharmacologists for drug design.[15–18] A second promising approach toward broadening our capacity to screen chemicals has also been increasingly used in pharmacological drug design: the development of so-called in silico models, or predictive-computational toxicology. Many approaches have been tried, from attempts to reproduce the logic of a working toxicologist through hierarchical sets of rules and decision trees, to correlating chemical structure or physicochemical properties with biological activity, to artificial intelligence systems such as neural networks that attempt to combine the best features of each approach.[19–21] The challenge is much greater for toxicologists than for drug designers, however, because identifying a single successful lead compound can make the approach successful for the latter, whereas missing a single toxic compound by the former could result in tragedy. Indeed, predictive models must continue to be developed through continuous interaction with traditional toxicologists, validating and extending models through targeted testing of new agents, and accounting for the greater considerations of the entire human organism and population.[22]

The second approach to defining exposures takes place in the field, often after an exposure has occurred or is suspected. Although this is the natural beginning of an epidemiological toxicological study, such physical measurements are traditionally the province of other practitioners, such as the industrial hygienist or the health physicist. Indeed, beyond the work environment or agents such as radon that are sometimes targeted by local health departments, often no attempt is made to measure or monitor the normal exposures that are thought to give rise to three-quarters of all cancer.[23] Besides the same potential problems with kinetics and interactions mentioned above, measuring genotoxicity in the field is complicated by the shear number of agents that a human being

or a population come into contact with, especially over the decades cancer may require to ensue. One approach has been to develop simple functional assays or *biosensors* that react to a spectrum of effectors rather than a single specific agent, such as a particular chemical. These instruments often use biological detectors, whole organisms or molecules such as antibodies or enzymes activated by interaction (binding) to xenobiotic agents to indicate the presence of such agents in the environment.[24] This approach is still restricted by our understanding of the underlying mechanism of action of such agents and, again, the application to cancer usually involves the assumption of a genotoxic mechanism, although methods to detect possible agents acting through an epigenetic hormonal mechanism have also been developed.[25]

## Biomarkers of Exposure: Internal Dose

To manifest a carcinogenic effect, most agents must be internalized within an organism and within a cell. *Biomonitoring* of potentially toxic exposures involves measuring the agent in a tissue or bodily fluid readily available for sampling.[26] In experimental systems, a potentially toxic substance can be labeled and administered to the whole animal by various methods, and the uptake, distribution, persistence, and elimination then investigated by recovery of the label in urine and feces. In potential human exposures, similar measures can be used to infer the magnitude and importance of the original dose. Such studies are complicated by the metabolism the original agent undergoes in vivo. Indeed, if the number and types of exposures humans normally undergo are daunting, the expansion of these effects through metabolism magnifies the problem many fold. In an effort to mobilize and detoxify potentially toxic substances, the body metabolizes or *biotransforms* them into more water-soluble derivatives; unfortunately, this often makes them more reactive and, therefore, more genotoxic, also, in effect, activating them. Thus, it is usually not only the original agent that must be monitored in bodily fluids, but also a complex mixture of metabolites that have different potentials for toxicity by themselves. This metabolism of chemical agents has become an important element in the individual exposure modification that must translate an exposure into a disease, and differences in the ability to metabolize chemicals have been shown to significantly affect their ultimate biological activity.[27] Most molecular epidemiological studies of genetic susceptibility to genotoxic agents have involved functional or genetic markers of metabolic enzymes.[28,29] Considering the number of potential phase I (esterases, cytochrome P-450 monooxygenases, epoxide hydratase, and so forth) and phase II enzymes involved in this process (methyltransferases, sulfotransferases, acetyltranferases, glucuronyl transferases, glutathione *S*-transferases, and so forth), it is difficult to predict the fate of a chemical in a biological system, although computational models have been developed.[30,31] There is an unfortunate tendency to look for associations between polymorphisms in these genes and health effects without ever determining whether the polymorphism has any effect on functionality. Since epidemiology can only be hypothesis-generating, demonstration of such an association should only provide further impetus for a functional analysis of the polymorphism and its mechanistic role in the disease process.[32,33]

## Biomarkers of Exposure: Biologically Effective Dose

Genetic toxicology is a unique subspecialty of toxicology in that the target molecule, DNA, is neither cell-type- nor organ-specific. Thus, a genotoxic effect, potentially contributing to carcinogenesis, can occur in almost any cell in the body. Certain non-genotoxic carcinogenic agents, such as transforming viruses and xenoestrogens are likely to be more restricted in the types of cells they can affect. Traditionally, genotoxicants have been defined rather narrowly as agents that interact directly with the DNA, although agents affecting chromatin proteins, microtubules, and so on, can affect DNA replication and chromosome segregation. Therefore, measurement of the effective dose of a carcinogen has often been done by quantifying DNA adducts (or blood protein adducts as a surrogate). There are many methods to do this in bulk, but the most widely applied is $^{32}$P-postlabeling, which yields "spots" of bases with altered migration in a two-dimensional chromatography system.[34–36]

An advantage of this and similar detection systems is that they quantitatively display all the base adduction products, so that all potential DNA damage can be estimated. The major disadvantage of such systems is that there are usually multiple species of adducted bases and, without individually characterizing each species, it is impossible to assign a relative importance to each spot. Although they must have a minimal persistence to be detectable at all (i.e., not removed from the DNA too quickly by DNA repair mechanisms), different altered bases can have very different effects on DNA replication and hydrogen bonding and, therefore, on the types and amounts of resulting mutations. Recent studies have often targeted a single, well-characterized adduction species with monoclonal antibodies; however, such studies assume that the total genotoxic effect of a mixed exposure can be estimated from a single mutagenic product, which is not likely to be consistent.[37,38]

## Biomarkers of Disease: Generalized Biological Effect

In keeping with the genotoxicity paradigm for carcinogenesis, the interaction of a toxic agent with DNA does not produce a long-term effect unless it results in an unrepairable mutation, defined as any heritable change in the amount or structure of the genetic material. Since we are referring to genetic changes in somatic cells, "heritable" suggests viable clonal propagation of the mutation through subsequent mitotic generations. A large number of methods for detecting and quantifying somatic mutation have been proposed and, to some degree, validated in retrospective studies.[39] Some markers, such as micronuclei or dicentric chromosomes, are inherently inviable; they therefore serve as indicators of similar processes that leave the cell mutated but alive (a sort of biomarker of a biomarker). Other monitored events, such as sister chromatid exchange, result in no genetic damage or biological effects, but are thought to respond to agents that can, in addition, induce chromosome breakage and rearrangement. The best validated biomarker of somatic mutation is the cytogenetic detection of stable chromosome aberrations, which has been shown to be predictive of subsequent cancer in three independent prospective studies.[40–42] These studies provide strong evidence that, although other processes may contribute to human carcinogenesis, induction of somatic mutation is an important factor in cancer incidence. Measurement of gene-specific mutation has also shown promise as an intermediate biomarker of biological effect.

### *Somatic Mutational Analysis*

There are two well-established methods for measuring gene-specific in vivo somatic mutation in humans. Both involve mutation at a non-oncogenic surrogate locus chosen to allow detection of mutation with single-hit kinetics. These well-characterized reporter genes are the X-linked gene coding for hypoxanthine-guanosine phosphoribosyl transferase (HPRT), a ubiquitously expressed purine scavenger enzyme, and the autosomal gene for erythrocyte glycophorin A (GPA), the most common sialoglycoprotein on the red cell surface, and the genetic determinant of the MN blood group. The HPRT gene has been used for many years as a selectable marker in mammalian cell culture,[43] and this assay system has been adapted to T-lymphocytes in short-term cultures derived from human peripheral blood.[44,45] The GPA assay is designed to detect a wide range of potentially inactivating mutations at the GPA locus by flow cytometric analysis of peripheral blood erythrocytes.[46,47] The two assays have complementary features (Table 1). The GPA assay is fast and inexpensive, using flow technology to quickly quantify rare mutational events. The HPRT assay requires cell culture and drug selection, making it more expensive and labor-intensive. However, the GPA assay can only be performed in genetically informative MN heterozygotes, and the mutational basis of the phenotypic variation cannot be confirmed at the molecular level, whereas the HPRT assay can be performed in virtually anyone, in a multitude of cell types, and can be used to generate mutational spectra that potentially can identify the inducing genotoxic agent. In previous studies using both assays, the correlation between these biomarkers is consistently better than the correlation of either with physical or environmental estimates of exposure, presumably because both of these assays consider the extent of exposure and the individual variations in response to genotoxic exposure.[48]

TABLE 1. Features of the GPA and HPRT In Vivo Human Somatic Mutation Assays*

| GPA | HPRT |
|---|---|
| Well-established assay, with extensive normals database | Well-established assay, with extensive normals database |
| Autosomal locus sensitive to mutational, chromosomal, and epigenetic events | X-linked locus sensitive to point mutation and small deletion |
| Applicable to only ~50% of the population | Applicable to everyone except patients with Lesch-Nyhan syndrome |
| <1 mL of fresh blood required | ~20 mL of fresh blood required |
| Inexpensive and rapid–direct-flow cytometric detection of mutants | Expensive and labor-intensive–cell culture and clonogenic drug selection |
| Mutant phenotype cannot be conformed at the DNA level | Mutant colonies can be genetically analyzed–generate mutational spectra |

* Adapted from Ref. 48.

The GPA and HPRT assays have been extensively validated as quantitative measures of genotoxic exposures. Investigations include exposures to ionizing radiation such as the survivors of the bombing of Hiroshima,[49–51] accidents such as Chernobyl,[52,53] and Goiânia,[54] and other medical,[55,56] environmental,[57] and occupational studies.[58,59] Similarly, the response of these systems to chemical exposures, such as PAHs and cigarette smoke has been established in a series of studies of environmental[60,61] and occupational exposures.[62–66] Given that these assays are sensitive to a wide range of genotoxicants, it has been suggested that these measures of somatic mutation might provide a biomarker of cancer risk associated with genotoxic exposure.[67,68]

There have been three studies specifically designed to determine whether newly diagnosed cancer patients have higher somatic mutation frequencies than disease-free individuals, that is, whether cancer incidence is associated with increased levels of gene-specific (as opposed to chromosomal) mutation. In 1989, a study of lung cancer patients with the HPRT assay demonstrated significantly higher mutant frequencies in the patient population versus controls.[69] A subsequent study of breast cancer patients revealed HPRT mutant frequencies higher than controls and women with benign breast masses, but the differences failed to reach statistical significance.[70] More recently, a significant increase in mutation at the GPA locus has been reported for a population of hepatocellular carcinoma patients.[71]

Several other mutational studies of cancer patients have been performed using the GPA assay, usually to demonstrate the genotoxicity of the therapeutic regimen.[72–75] Our studies of this type have always involved analysis of both concurrent disease-free controls and a pre-therapy sample from each patient. When the results from these two populations are pooled and compared, the patients are significantly higher for total variant frequency (combining both allele-loss and loss-and-duplication classes) ($p < 0.01$) (Fig. 2). These data include subpopulations of patients with breast,[76] prostate,[56] and testicular cancer.[77] The HPRT assay has also been used extensively to demonstrate a genotoxic effect of cancer chemotherapy upon circulating lymphocytes. In addition to the two mentioned above, seven other studies have been published in which the frequency of lymphocytes with mutations at the X-linked HPRT locus was determined in newly diagnosed cancer patients before genotoxic therapy.[78–85] In all nine studies, the frequency of somatic mutation at the HPRT locus was higher in the cancer patients than in concurrent controls. When these data were reviewed and pooled for re-analysis,[86] the approximately twofold elevation in somatic mutation frequency demonstrated by these pooled data from cancer patients ($N = 187$) was highly significant ($p < 0.001$) (Fig. 3).

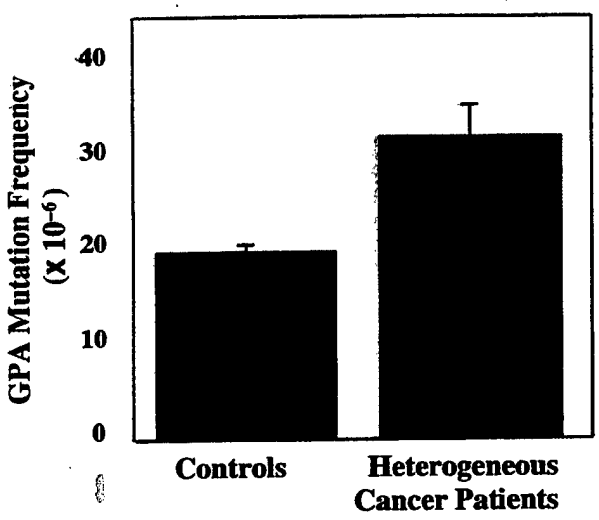


FIGURE 2. Comparison of in vivo somatic mutation at the GPA locus in a population of untreated patients with diverse types of cancer and disease-free controls.

These data suggest that human carcinogenesis is associated with increased in vivo somatic mutation and, based on the validation studies detailed above, that these mutation assays could act as integrative biodosimeters for genotoxic exposures. It is significant that the association seems to hold not just in tumors with a well-accepted mutagenic etiology, such as lung cancer, but also in tumor types with viral (hepatocarcinoma) or hormonal (breast, testicular, prostate cancer) components in their progression. This observation is consistent with the concept of a multistep mutational pathway of carcinogenicity in which one or a few steps can be fulfilled by epigenetic factors, but numerous other steps still depend on mutagenesis. These assays can measure both transient and persistent DNA damage in the stem cell and differentiating hematopoeitic compartments, respectively, and show great promise as biomonitors of chemopreventive measures against genotoxicity, such as antioxidants.

The association of cancer incidence with a modest elevation in somatic mutant frequencies suggests that cancer can be caused by normal or background levels of genotoxic exposure. Individual variation in susceptibility to genotoxic insults would therefore become an important factor in determining whether mutagenesis and, subsequently, carcinogenesis would result from a particular exposure. The HPRT and GPA assays have also been applied to individuals and populations suffering from DNA repair-deficiency syndromes, which are characterized by very high cancer incidences. Thus, HPRT mutation has been found to be spontaneously elevated in homozygotes for the recessive cancer-prone disorders Bloom syndrome,[87] Fanconi anemia,[88,89] and ataxia telangiectasia,[90,91] all associated with deficiencies in resolving DNA double-strand breaks. The GPA assay has demonstrated 10-(ataxia telangiectasia), 50-(Fanconi anemia), and 100-fold (Bloom syndrome) increases in the frequency of spontaneous somatic mutation in these patients.[74,89,92–95] HPRT mutant frequencies appear to be elevated in xeroderma pigmentosum patients, which are characterized by a deficiency in nucleotide excision repair,[96,97] but there is no evidence for such an increase at the GPA locus.[98] Both assays have demonstrated subtle elevations in mutant frequency in the premature-aging disease, Werner syndrome.[99,100] These studies offer an alternative explanation for the elevated mutation frequencies observed in the sporadic cancer patient populations described above; namely, instead of sustaining slightly higher than normal genotoxic exposures, these individuals manifest slightly higher than normal genetic susceptibilities to genotoxic injury. This suggestion is similar

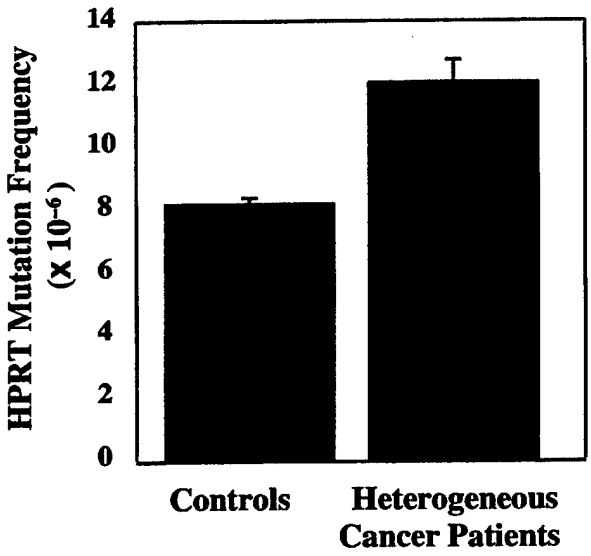


FIGURE 3. Comparison of in vivo somatic mutation at the HPRT locus in a population of untreated patients with diverse types of cancer and disease-free controls.

to the proposal by Hsu[101]: Normal populations should show interindividual variability in DNA repair capacities, and those with the highest susceptibility to unavoidable genotoxic exposures, but still within the range of normal, would be at greatest risk of developing cancer.

### *Mutagen Sensitivity*

Hsu's own approach to demonstrating this principle was based on another characteristic of the cancer-prone syndromes: their hypersensitivity to DNA-damaging agents.[102–104] This cellular phenotype has been exploited to map and clone the underlying genes responsible for these conditions, and lymphocyte mutagen hypersensitivity continues to be used as a definitive diagnostic laboratory test. Hsu conjectured that milder forms of this mutagen-sensitivity phenotype should occur in the human population, and might contribute to the incidence of common tumors in the normal population. He adapted the mutagen-sensitivity tests developed for diagnosis of the DNA repair-deficiency diseases into a screening tool based on the induction of transient cytogenetically detectable chromatid breaks.[105–108] These studies demonstrated significant interindividual variation in the response of the disease-free population to a known genotoxic agent, the radiomimetic DNA cross-linking agent, bleomycin. They also demonstrated that a significantly greater proportion of individuals manifesting a number of different types of cancer were hypersensitive to this mutagen in that they suffered more DNA damage when their lymphocytes were exposed to a standard dose of bleomycin. This work has been carried forward by Spitz et al.,[109,110] in a series of studies demonstrating that bleomycin sensitivity is associated with risk of head and neck[109–111] and lung[112,113] cancer. Hsu et al.[114,115] introduced the idea that sensitivity to other mutagenic chemicals could also be measured by induction of chromatid breaks. In these studies, the inducing agent was 4-nitroquinoline-1-oxide (4NQO), which causes the same type of DNA damage as UV light, the genotoxic agent implicated in skin carcinogenesis. This principle has subsequently been applied in the lung cancer study using the polyaromatic hydrocarbon and tobacco smoke mutagen benzo[*a*]pyrene diol epoxide (BPDE) as the inducing agent.[116,117]

## Biomarkers of Disease: Specific Biological Effect

Just as some would argue that an adduct is not important unless it results in a mutation, others would argue that the mutation is not important unless it is involved in the progression of the disease. Screening for mutations in oncogenes and segregation of tumor suppressor genes[118] blurs the distinctions of public health concerns, such as identifying individuals at increased risk of cancer, and purely medical concerns, that is, the early detection of the disease itself. Whatever the intent of the study, it can take the form of a screen because of the early observation that tumor cells (and potentially preneoplastic cells as well) can be found in many fluids and excreta of the body.[119] Advances in cytological techniques and the development of antibodies to cell lineage markers and carcinoembryonic antigens maintained interest in these cells, but the possibility of molecular screening only arose with the delineation of the role of somatic mutation in oncogenesis. Thus, there has been much interest and some progress in the past decade toward using molecular detection of so-called early mutations in such biological samples as buccal swabs, mouth rinses, lung lavage, urine, feces, and so on, as diagnostic and prognostic markers.[120] More recently, it has been found that free circulating DNA in serum, long known to be at higher levels in cancer patients,[121,122] is primarily derived from necrosing and apoptosing cells.[123,124] Activated oncogenes[125,126] and segregated tumor suppressor genes,[127,128] reflective of genetic changes in the primary tumor, have been detected by analysis of DNA amplified from blood samples from cancer patients.

## Conclusion

In many ways, the fields of molecular toxicology and molecular epidemiology are in a holding pattern. There has been a general reluctance to leave the validation phase in which potential biomarkers are evaluated in populations with known—and usually extreme—exposures and predispositions to cancer and to move these studies into the general population, and, subsequently, into clinical or public health practice. For basic scientists, this involves taking on responsibilities for interaction with human populations

and individuals that some researchers may not appreciate. From the clinical side, in the absence of an established intervention, there may be little reason or even justification for predicting disease. Only through applying the biomarkers that do exist, such as the promising technologies discussed in this report, can basic scientists become comfortable with such translational research and can preventive measures be developed to provide practitioners with an armamentarium to treat preneoplastic disease.

The biomarkers available and in development for cancer reflect, to a large degree, the inclinations of toxicologists to equate mutagenesis with carcinogenesis. Indeed, data presented here suggest that although other mechanisms are known to contribute to cancer, mutation, both chromosomal and gene-specific, appears to be involved in all cancers. This, in turn, suggests that (1) if one lives long enough, one will inevitably develop cancer, and (2) the specific type of cancer will be that which one is most susceptible to owing to the types of exposures sustained as well as one's underlying genotype. Thus, a certain level of genotoxic effect may be sufficient to cause hepatocarcinoma in an individual with a chronic hepatitis B infection, but a slightly later onset of kidney tumor in an individual without such a viral predisposing factor. The strength of the mutational model of cancer lies in its ability to rationalize itself with these other factors. As mentioned above, we know that viruses can deliver activated oncogenes (retroviruses), or provide a protein sink for tumor suppressor gene products (animal viruses).[129] Genetic factors in cancer have been found to congenitally provide a mutation that traverses one step in the carcinogenic pathway,[130] or confer a mutatorphenotype that causes a more rapid progression through the pathway,[131] or both. Hormonal factors, even if they are not overtly genotoxic, can mimic mutation via their effects on transcriptional regulation,[132] or affect mutation rates as suggested by the mitogen-mutagen hypothesis.[133] Toxicologists must also be willing to expand their definition of a mutagen; for example, because aneuploidy is unquestionably a mutational event, agents that cause it through interaction with centromeric proteins or microtubules (as opposed to direct interaction with DNA) should be considered mutagens. Despite the present success of mutationally based biomarkers, we must be aware that application of such surrogate end points for cancer depends on the confidence the entire field feels in the underlying mechanism of cancer. Some clinicians maintain that the only credible intermediate biomarker for carcinogenesis, especially for prospective trials of chemoprevention, is the appearance of preneoplastic lesions[134]; this despite the fact that the vast majority of such lesions do not, and perhaps cannot, develop into malignant tumors.[135] Mutational biomarkers have also been criticized for not discriminating between exposure and susceptibility, or for not being more agent-specific. The best reply to such criticisms is to apply the markers we have now in the most appropriate way and, if such discrimination is found important, to continue to develop methods to further specify the relative contributions of each factor in each particular disease or lesion.

All of the preceding is based on the assumption that toxicology, pathology, and so forth, will continue playing an important role in oncology. History, however, suggests that medical science tends to follow the fad of the latest technology, even when it is not necessarily appropriate. With the recent completion of the first phase of the human genome project, we have entered into a period of increased enthusiasm for genomic research that may or may not complement the types of research discussed in this report. We mentioned earlier that every cell from 80-year-old subjects still carries their entire genetic code, facilitating such genomic research, even in such late-onset diseases as cancer. Our 80-year-olds also have a complete record of their lifetime of accumulated exposures, at least genotoxic exposures, in their cells, although different aspects may be found in different cell types, locations, and so forth. We must develop methods of rapidly screening individuals for evidence of cumulative past exposures that can be used to characterize their levels of response. The justification most often given for the extensive involvement of the U.S. Department of Energy in the human genome project was, essentially, how could we identify mutations unless we know what the normal gene sequence is? We now need to take up this challenge and use the technologies developed for charting the evolution of the hereditary genome through generations to begin to map the changes in the somatic genome that occur over a normal lifetime and during the carcinogenic process.

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